Method for the Analysis of Thyreostats in Meat Tissue Using Gas Chromatography with Nitrogen Phosphorus Detection and Tandem Mass Spectrometric Confirmation

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Abstract

An analytical method not requiring a mercury column cleanup step is described for the isolation and detection of four thyreostatic agents in meat tissue. The use of these growth promotants in livestock has been banned by regulatory agencies. The meat tissue is homogenized with acetonitrile–water, centrifuged, and the supernatant is partitioned with petroleum ether. The acetonitrile–water is concentrated and then passed through a silica-gel column. The solvent is then removed and the residue derivatized with *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide. The total amount of organic solvent used for the analysis is merely 35 mL. The derivatized thyreostats are detected and quantitated by gas chromatography (GC) equipped with a nitrogen–phosphorus detector. Percent recoveries from fortified meat tissue (n = 6) at the 0.1-µg/g (parts per million) level are 93.5 ± 2.9 for 2-thiouracil, 90.3 ± 3.0 for tapazole, 87.5 ± 2.9 for 6-methyl-2-thiouracil, and 85.1 ± 5.8 for 6-*n*-propyl-2-thiouracil. For the confirmation of analyte identities, GC–tandem mass spectrometry with an ion-trap instrument is used. The estimated minimum level for a reliable measurement is 0.050 µg/g in meat tissue.

Introduction

Thyreostatic or goitrogenic agents that inhibit the production of thyroid hormones can be used to increase the weight of animals prior to slaughter by enhancing water retention. Although their use is prohibited in most countries because of their potential adverse health effects and their adverse effect on meat quality, they are still used illegally. As a result, methods are needed to determine if residues of these compounds are present in the food supply. Thyroid inhibitors (TIs) are comprised primarily of 2-thiouracil (TU), and its derivatives are substituted at the 6-position with either a phenyl group (PhTU) or alkyl groups such as methyl (MTU) and propyluracil (PTU). TIs also include 1-methyl-2-mercapto-imidazole—also known as tapazole (TAP)—and 2-mercapto-benzimidazole (MBI). The isolation of these compounds from tissue is problematic because they are polar and hence hydrophilic. Most published analytical methods are for the determination of TIs in urine, which is where the highest residues occur other than in the thyroid itself (1).

Like most analytical methods used for biological tissues, solvent is added to homogenize and blend the tissue, then the mixture is centrifuged before conducting solvent concentration and chromatographic analyses. In the case of animal-derived tissue, the procedure usually requires a defatting and extract cleanup step. The most common means of TI isolation and cleanup is to form a mercuric complex of the TIs on a mercurated ion-exchange column and then elute the TIs from the column (2). In this method, the TIs are analyzed using thin-layer chromatography (TLC) with fluorescence detection (2) or by gas chromatography (GC)–electron-capture detection after derivatization with pentafluorobenzoyl chloride (1). Alternatively, GC with mass spectrometric (MS) detection can be used for TI confirmation after a trimethylsilylation step (2). This method has been adopted by the European Union (EU) for the analysis of these compounds (3). However, because of the hazards associated with the use of mercury-containing reagents, it would be highly desirable to eliminate their use from any analytical procedure.

Reported alternatives for mercurated resins include adsorption of the TIs onto ion-exchange columns followed by TLC-fluorescence detection (4) or methylation followed by GC–flame photometric (5) or GC–MS (6) detection. In both of the latter cases, the TIs on the column are reacted with methyl iodide to form their more volatile 2-methylthio-3-*N*-methyl derivatives. Yu et al. also conducted in situ methylation with methyl iodide during supercritical fluid extraction (SFE) and obtained comparable results (6).

The application of liquid chromatography (LC) with UV detection or the more selective electrochemical detection was applied to TU and MTU in meat tissue (7). More recently, LC–MS was used for the detection of TIs in thyroid tissue (8). Derivatization was not needed in the LC methods, and recovery of TIs in general was good (except for TU, which was < 50%) (8). In both LC methods, the extracts were subjected to silica-gel solid-phase extraction (SPE) cartridges, indicating that alternate cleanup approaches are feasible.

To eliminate the use of the mercury-containing affinity

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(Figure 1, standard 1, peak at 24.681 min), but recoveries averaging < 35% prevented its further inclusion. At the 0.1 µg/g fortification level, recoveries for the four remaining TIs ranged from 85.1% for PTU to 93.5% for TU. These recoveries compared favorably with the mercurated resin cleanup method (1) and are superior to an LC method (12), which reported recoveries < 70% at the 0.15-µg/g level for the underivatized analyte.

Typical chromatograms obtained by using this method with GC–NPD are shown in Figure 1. The top chromatogram shows all six of the TIs in solution at a level equivalent to 0.5 µg/g. The second chromatogram shows the four TIs analyzed at the 0.1-µg/g equivalent level in a standard. The third and fourth chromatograms are a control and fortified sample, respectively, at the 0.1-µg/g level. We found that the number of interfering peaks increased over time and derivatized analytes degraded, even when the extracts were stored at –20°C. Therefore, either fresh meat samples were analyzed soon after extraction or the final extracts were stored at –85°C, which reduced degradation.

The analysis of residues (especially those banned for use in food producing animals) runs the risk of false positives or negatives. In order to eliminate this potential, a second method is required in regulatory analysis for residue confirmation. In this study, the four TIs in 0.1-µg/g fortified meat extracts were confirmed using GC–MS–MS. The use of GC–MS with EI alone only produced a single ion of significance for the TIs, except for tapazole which gave two ions (171 and 186 *m/z*). Therefore, GC–MS with EI was insufficient for confirmation, which generally requires three ions. In MS–MS, the single ion in the EI–MS spectrum can be isolated and further dissociated to permit confirmation using two product ions in the proper ratios. As shown in Figures 2 and 3, this approach was used in the confirmation of TIs in the 0.1-µg/g fortified meat samples. High signal-to-noise ratios were obtained for the TI peaks in the product-ion chromatogram despite the presence of other coeluting matrix components that were observed in GC–MS chromatograms of the meat extracts. The corresponding MS–MS spectra matched closely with the spectra obtained from the TI standards.

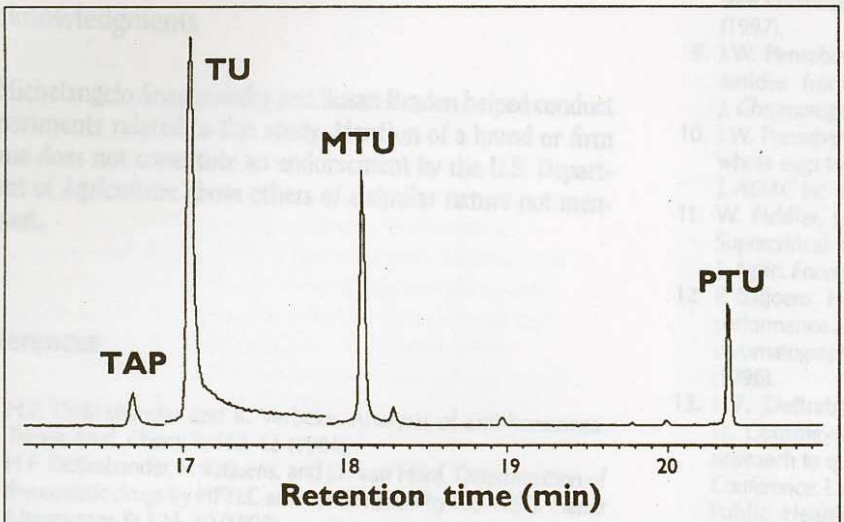

Figure 2. GC–MS–MS chromatogram of product ions for four thyreostats in the extract fortified at 0.1 µg/g in meat.

For banned veterinary substances in food such as TIs, the EU has recommended a 4-point identification system for MS confirmation (13). In this identification system, each ion in low-resolution MS is worth 1 identification point and each product ion in MS–MS is 1.5 points. Thus, the two product ions in combination with the isolated parent ion is worth 4 points and meets the confirmation criteria for banned veterinary drugs by the EU.

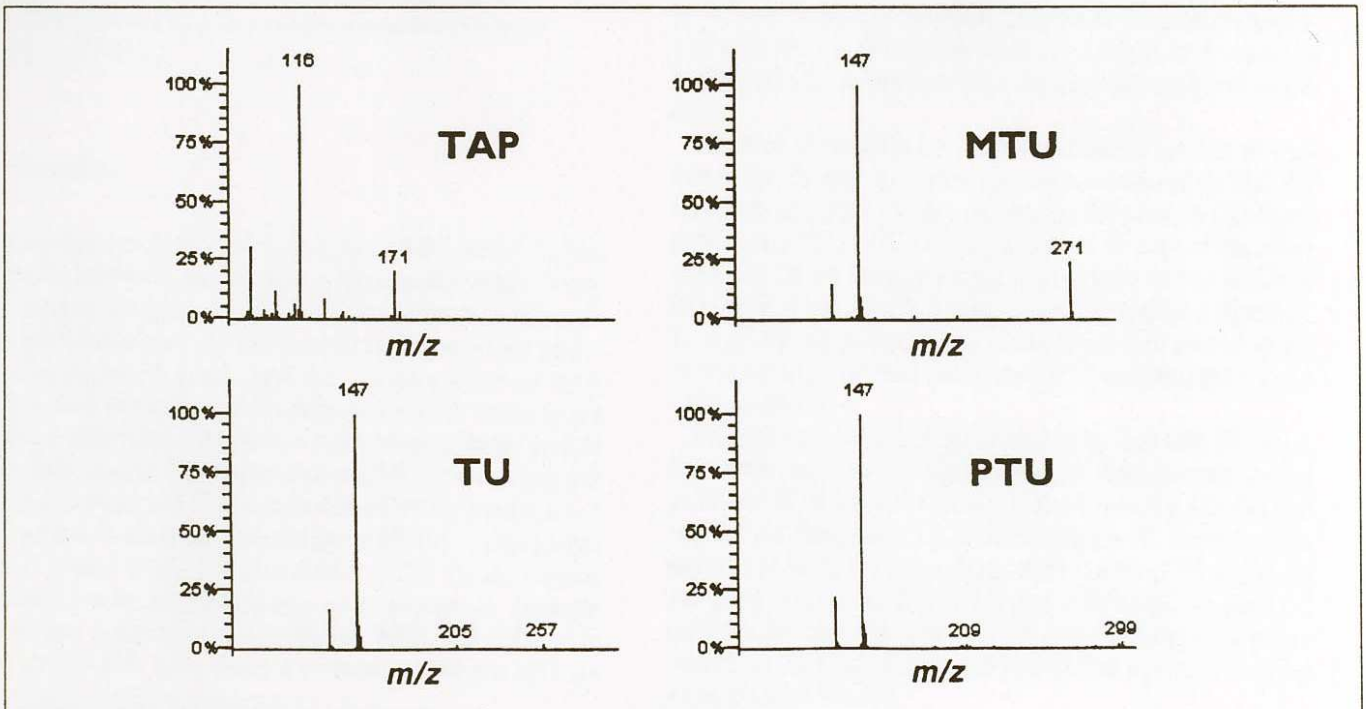

Figure 3. The MS–MS spectra of four thyreostats in the extract fortified at 0.1 µg/g in meat.

product ions while still leaving some of the precursor ion in the spectrum. This optimized MS–MS spectrum provided the acceptable level of confirmation while maintaining a high signal-to-noise ratio for the trace TI residues in the tissue extracts. Table I lists the retention times and optimized MS–MS parameters used for confirmation of the TIs.

Results and Discussion

With the current emphasis on reducing hazardous waste generation, the use of SFE with carbon dioxide has been evaluated for residue analysis (9–11). Although it is effective for some nonpolar and intermediate-polar drug residues, there is no evidence for its use in TI analysis except for Yu et al., who used it for in situ derivatization of TIs. We hypothesized that SFE has the potential for yielding clean extracts without the need to use the mercurated affinity column used by EU member countries (3) among others. However, we found that SFE at 10,000 psi (680 bar) and at temperatures up to 100°C with supercritical carbon dioxide ($SC\text{-}CO_2$) alone was not effective in extracting TIs from fortified tissue. In SFE, the addition of a small amount of cosolvent to the $SC\text{-}CO_2$ can frequently help overcome analyte–matrix binding and more readily solubilize polar analytes. However, the use of a 2.5 and 5.0% methanol modifier (added to the $SC\text{-}CO_2$ via a syringe pump) gave a sample extract with a large number of interfering peaks (even after cleanup on a silica-gel SPE cartridge) when analyzed by GC-NPD after derivatization. In an attempt to improve the selectivity of this method, in situ methylation with methyl iodide (6) mixed with Hydromatrix in the SFE vessel was studied, but recoveries (usually < 60%) were obtained. Clearly, an approach other than SFE to eliminate the mercurated cleanup column was necessary.

The use of a silica-gel cleanup step has recently been shown to be effective for determining TIs in thyroid tissue extracts by both LC–MS (8) and high-performance LC–UV (3). We sought to combine this cleanup step (after TI extraction with acetonitrile) with trimethyl-silylation derivatization using MSTFA, an approach previously used for tissue (2) and urine (12). MSTFA forms volatile TI derivatives, which allows for the use of a more selective and sensitive GC approach with a selective NPD detector. This approach also allows for the easier confirmation of apparent positive samples by GC–MS–MS compared with LC or TLC techniques. We also attempted to use a silver nitrate impregnated silica-gel column for cleanup rather than the mercurated affinity column, but we found that the extracts could not be quantitated after derivatization because of the large number of interfering peaks present in the GC–NPD chromatogram.

Various solvents (ethyl acetate, acetone, methanol, and water) were tried in addition to acetonitrile to determine their effectiveness in extracting TIs from meat samples. However, only acetonitrile was effective in extracting all of the TIs from the various fortified meat samples examined in this study. Solvents more polar than acetonitrile (although equally effective for extraction) increased the number of coextractants and caused detection and quantitation problems. However, during these experiments we found that the addition of 1.0 mL of water to the acetonitrile extracts improved the liquid–liquid partitioning step with the petroleum ether and resulted in consistently higher yields of the TIs.

Recovery studies were carried out on beef or pork tissue at fortification levels of 0.1, 0.5, and 1.0 µg/g ($n = 6$ at each level), and the results are given in Table II. At the 1.0- and 0.5-µg/g level, the tissue was fortified with TU, TAP, MTU, PTU, and PhTU. Because of its poor GC–NPD response, derivatized PhTU (Figure 1, standard 1, peak at 26.12 min) could not be detected below 0.5 µg/g. In addition, MBI was initially included in this procedure

Table II. Thyreostat Average Percent Recoveries and Repeatability* from Fortified Meat Tissue Samples†

Analyte	Fortification level (µg/g) 1.0	0.5	0.1
TAP	102% (3%)	98% (3%)	90% (3%)
TU	102% (4%)	102% (3%)	94% (3%)
MTU	98% (3%)	94% (4%)	88% (3%)
PTU	97% (5%)	92% (5%)	85% (6%)
PhTU	88% (6%)	86% (5%)	not performed

* $n = 6$ at each level.
† Percent relative standard deviation.

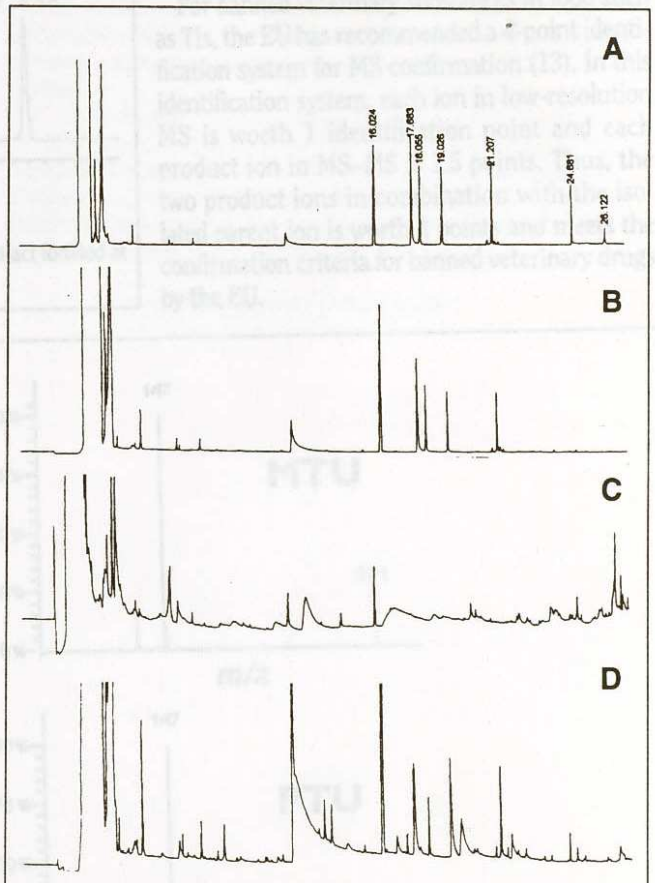

Figure 1. GP–NPD chromatograms showing: (A) standard 1 consisting of MSTFA at 16.02 min, TAP at 17.68 min, TU at 18.05 min, MTU at 19.02 min, PTU at 21.20 min, MBI at 24.68 min, and PhTU at 26.12 min at the 0.5-µg/g level; (B) standard 2 consisting of MSTFA, TAP, TU, MTU, and PTU at the 0.1-µg/g level; (C) a control meat sample; and (D) a fortified meat sample at the 0.1-µg/g level.

column, we also investigated the use of silica-gel cleanup. In this study, we present a simple multiresidue GC–nitrogen–phosphorus detection (NPD) method applicable to muscle, kidney, thyroid, and liver tissues using only a minimum amount (35 mL) of organic solvent. Furthermore, GC–MS–MS conditions are developed to confirm the TIs present in the extracts.

Experimental

Materials

Beef and pork samples were obtained from local supermarkets. The meat was ground twice, thoroughly mixed to insure a representative sample, and then stored at –20°C until analyzed. All samples were analyzed prior to the fortification studies in order to insure the absence of any thyreostatic agents. TU, PTU, and PhTU were purchased from Sigma (St. Louis, MO). TAP and MTU were purchased from Lancaster Chemicals (Pelham, NH) and MBI from Aldrich Chemical (Milwaukee, WI). Stock solutions containing 200 µg/mL of each thyreostat were prepared biweekly in methanol and stored at –20°C. Standard solutions were prepared from the stock solution as needed. Acetonitrile, petroleum ether, methanol, acetone, and dichloromethane (DCM) were of a Burdick and Jackson brand purchased from AlliedSignal (Muskegon, MI). Anhydrous sodium sulfate was purchased from Fisher Scientific (Fair Lawn, NJ), and the 1-g silica-gel cleanup SPE columns were purchased from United Chemical Technologies (Bristol, PA). The *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) was purchased from Sigma.

Sample extraction

Comminuted meat (5.0 g) was weighed into a 50-mL polypropylene centrifuge tube, and 25 µL of the appropriate standard solution was added to achieve fortification levels of 1.0, 0.5, and 0.1 µg/g. Fortified samples were allowed to equilibrate at room temperature for 15 min prior to the addition of solvent; 10 mL of acetonitrile and 1.0 mL of water were added to the tube. The contents were homogenized for 1.0 min using a Tissuemizer (Tekmar, Cincinnati, OH) equipped with an S25N-100 blade. The Tissuemizer blade was rinsed with 1 mL of acetonitrile, then the tube was placed into a Sorvall RT6000B refrigerated centrifuge (Dupont, Newtown, CT) and centrifuged at 3000 rpm for 10 min at 10°C. After centrifugation, the liquid was decanted into a 60-mL separatory funnel containing 10 mL of petroleum ether. The funnel was shaken for 2 min, the lower acetonitrile layer collected in a 10-mL concentrator tube (Kontes, Vineland, NJ), and the upper petroleum ether layer discarded. The liquid was concentrated in a 50°C water bath to approximately 0.5–0.8 mL under a stream of nitrogen gas in a N-Evap analytical evaporator (Organomation, Northborough, MA). A 1.0-g silica-gel SPE cartridge (to which 4.0 g of anhydrous sodium sulfate was added) was prewashed with 4 mL DCM. The sample extract was transferred to the silica-gel column, the tube vortexed twice with DCM (2 × 1 mL), and the washes added to the column. The silica gel was washed with 4 mL DCM and the thyreostats eluted from the column with 4 mL of 25% methanol (v/v) in DCM. The eluent was collected in a 4-mL concentrator tube, the solvent evaporated to dryness under a stream of nitrogen gas in a 50°C water bath, and 100 µL of MSTFA added. The tube was heated at 55°C for 30 min. After derivatization, the tube was removed from the water bath, diluted to 1.0 mL with acetone, and transferred to an autoinjector vial for detection and quantitation by GC–NPD and confirmation by GC–MS–MS.

Table I. Optimized MS–MS Parameters Used for Confirmation of the Thyroid Inhibitors in Extracts

Analyte	Retention time (min)	Parent ion (*m/z*)	Excitation storage level (*m/z*)	Excitation amplitude (V)	Product ions (*m/z*)
TAP	16.69	171	75	80	116, 88
TU	17.06	257	100	90	147, 131
MTU	18.10	271	100	87	147, 131
PTU	20.39	299	100	88	147, 131

GC–NPD analysis

GC–NPD analyses were carried out using a Hewlett Packard 5890 Series II GC (Agilent Technologies, Wilmington, DE) equipped with a splitless capillary injector and an NPD. The sample extracts were injected (2.0 µL) onto a DB-5MS capillary column (30-m × 0.25-mm i.d., 0.25-µm film thickness) (J&W Scientific, Folsom, CA) using a Hewlett Packard 6890 series autoinjector. The GC oven program was as follows: 80°C for 2 min; raised from 80°C to 150°C at 5°C/min; raised from 150°C to 280°C at 10°C/min; and then held at 280°C for 6 min. The injector was held at 250°C and the detector at 300°C. The carrier-gas linear velocity (constant flow) was set at 32.9 cm/s (1.35 mL/min) with an He makeup gas flow rate of 30 mL/min. The injector purge was activated at 1.0 min and shut off at 1.5 min from the start of the injection. The minimum level of a reliable TI measurement based on the signal-to-noise ratio of 2 to 1 was calculated to be 0.050 µg/g. During the course of these analyses, occasional maintenance was required when extraneous peaks started to appear in the chromatogram. This was solved by removing a 15-cm section at the front of the capillary column, thereby slightly changing the retention times of the TIs.

GC–MS–MS confirmation

A Saturn 2000 (Varian, Walnut Creek, CA) GC–ion-trap MS–MS was used to confirm the TIs in the extracts. Similar chromatographic conditions were used for GC–NPD with the only exception being the use of a 1.2-mL/min constant flow rate for an Rtx-5MS column (30-m × 0.25-mm i.d., 0.25-µm film thickness) (Restek, Bellefont, PA). Temperatures of the ion trap, manifold, and transfer line were 150°C, 50°C, and 250°C, respectively. The isolation windows were 3 *m/z* for each parent ion, and data collection rates were set to give 4 µscans per data point (approximately 0.6 s) for a scan range starting from 5 *m/z* units below the lowest product ion and above the precursor ion. Electron ionization (EI) with a 50-µA filament current and nonresonant collision-induced dissociation were used in the MS–MS method rather than a previous approach for ion-trap MS using positive chemical ionization with isobutane (2). The MS–MS conditions were optimized to give the highest signal of two

Conclusion

We have developed a method that isolates, quantitates, and confirms thyreostatic residues in meat tissue. The method uses solvent extraction followed by silica-gel SPE cleanup. It gives excellent recoveries and reproducibility, uses minimal amounts of organic solvent, and permits the analysis of 4–8 samples for each day per analyst. It also has distinct advantages over commonly used methods in that it eliminates the use of the mercurated affinity cleanup column, thereby generating less hazardous waste for disposal. Conditions for the GC–MS–MS analysis of TIs were also developed and demonstrated to give an unambiguous identification of the targeted TIs below 0.1 µg/g in fortified meat.

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